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(6) EXPERIMENTAL CHEMOTHERAPY: A RAPID AND SIMPLE
/// SCREENING METHOD FOR DRUG BINDING TO DNA

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I. BACKGROUND

Histories of wars and of infectious diseases have been inter-
woven since the time of antiquity. In World War II, some 500,000 Ameri-
can servicemen acquired malaria with an attending loss of 6.6 millions
of man days. During 1965, the number of American soldiers evacuated
from Vietnam because of chloroquine-resistant malaria, equalled the
number evacuated because of wounds (1). The invasion of Taiwan from
mainland China, planned in 1949, had to be abandoned because of a cata-
strophic outbreak of schistosomiasis which the assembled troops acqui-
red while practicing landing maneuvers on inland lakes in Fukien pro-
vince. Earlier, the campaign of Napoleon in Egypt faltered because of
schistosomiasis and trachoma in the expeditionary force.

Drugs for the treatment of those communicable diseases a-
gainst which there exists no effective immunoprophylaxis are a military
necessity when troops must be deployed in unsanitary parts of the
world. The Russian Civil War (1917-1924) was accompanied by 25,000,000
cases of epidemic typhus. Today, such patients would be treated success-
fully with chloramphenicol or tetracyclines.

In the preparation for warfare, the development of chemothe-
rapeutic drugs is an absolute necessity. Search for, and development of,
such drugs still employs empirical methods. The discovery of lead com-
pounds and their successful molecular modification would be facilitated
by the introduction of exact scientific pre-screens. This article de-
scribes such a screening method.

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II. SCIENTIFIC INTRODUCTION

Numerous drugs against parasitic diseases, such as malaria, schistosomiasis, trypanosomiasis, and leishmaniasis form complexes with DNA (Table 1). DNA-binding compounds are among inhibitors of nucleic acid polymerase reactions of DNA viruses and retroviruses (Table 2) and furnish leads to the development of virus chemotherapy (2). The largest number of DNA-complexing drugs is found among anticancer compounds (Table 3). DNA-complexing compounds also eliminate drug-resistance plasmids from cultures of multiresistant bacteria and, hence, restore sensitivity of such organisms to antibiotic and synthetic drugs (3,4). The agents in Tables 1 - 3 act as DNA template poisons that inhibit the replication of DNA and/or the transcription of RNA from DNA.

Table 1. DNA-Complexing Antiparasitic Drugs

Acriflavine	Hydroxystilbamidine
Berberine	Miracil D
Berenil	Pentamidine
Chloroquine	Propamidine
Congocidine	Quinine
Ethidium Bromide	Quinacrine
Hycanthone	Stilbamidine

Table 2. DNA-Complexing Compounds with Antiviral Action

Distamycin A	Nitroacridine C-283
Ethidium Bromide	Tilorone

Table 3. DNA-Complexing Antitumor Compounds

Adriamycin	Echinomycin
Anthramycin	Ellipticine
Bleomycin	Mithramycin
Chromomycin	Nitroacridine C-283
Daunomycin	Nogalamycin
Dichloro-diammino platinum	Olivomycin
Distamycin A	Sibiromycin

DNA-binding compounds can be designed (5). They are also found among antibiotics (Table 3) as well as among empirically discovered synthetic drugs (Table 1). Since the ability of a compound to form a molecular complex with DNA signals potential chemotherapeutic

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activity, it follows that an _in vitro_ pre-screen for DNA binding will be a useful step in experimental chemotherapy programs.

The subject of this article is a simple, rapid, and inexpensive screening procedure for drug binding to DNA. It yields numerical results which are proportional to biochemical and biological activities of the tested compounds. The method is based upon the displacement of methyl green from its complex with duplex DNA. It requires only a simple spectrophotometer and the (commercially available) methyl green-DNA reagent.

III. METHYL GREEN AND ITS COMPLEX WITH DNA

Methyl green, MG, is a basic triphenylmethane dye (Fig. 1). It is used as a histochemical stain for DNA. The DNA-MG reagent is an experimental substrate for the determination of deoxyribonucleases (6. 7). The first qualitative observation of a displacement of MG from DNA by drugs was made for chloroquine and quinacrine (8).

The use of the DNA-MG complex in displacement analysis is based upon the fact that free MG in polar solutions of pH >5 undergoes spontaneous molecular rearrangement to its colorless carbinol base (9) so that the liberation of the dye from DNA can be followed spectrophotometrically as an exponential decrease in absorbance at 640 nm with a first order rate constant of $0.65 \times hr^{-1}$ (10). In its complex with DNA, MG is stabilized against molecular rearrangement and, hence, maintains its color.

In order to understand the molecular mechanism of the displacement of MG, its complex with DNA has been studied (10). MG binds to double-stranded (calf thymus) DNA with a stoichiometry of one dye mole-
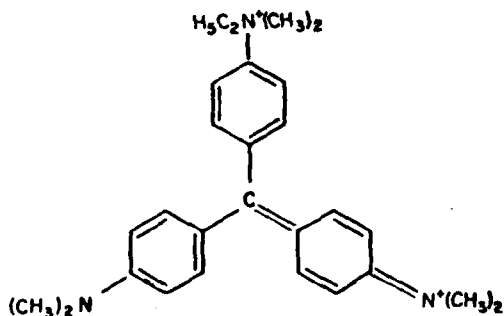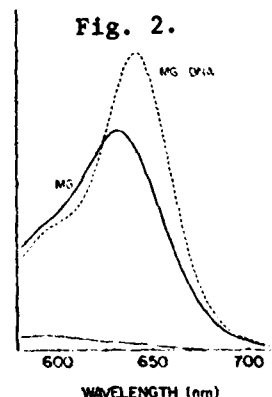

Fig. 1.   METHYL GREEN

Fig. 2.

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cule per 13 nucleotides (9). This is attended by a bathochromic shift of 10 nm in the absorption maximum of MG and a hyperchromic increase of 40 per cent in this maximum (Fig. 2). Bathochromic shifts indicate the binding of single dye molecules rather than of dimers or aggregates (11). Hyperchromicity suggests peripheral binding to DNA rather than insertion between base pairs (intercalation) in the interior of the double helix: intercalation places chromophores into a less polar environment and, thus, produces hypochromic changes in their absorption spectra.

The naturally single-stranded DNA of ϕX174 also causes the bathochromic shift in the absorption spectrum of MG but does not produce hyperchromicity. After ϕX174 DNA and MG are combined in solution, the absorption of the dye decreases with a time course, similar to that of free MG. A small portion of MG remains stably bound to this single-stranded DNA. (10).

The synthetic duplex DNA-like polymer, poly[d(A-T)], causes the same manifestations as DNA in the absorption spectrum of MG and also stabilizes the dye. In contrast, poly(dG-dC) produces a slight hypochromic change at 640 nm, followed by slow decolorization with an absolute endpoint of 33 per cent of MG remaining stably bound to the polymer (10).

Besides by base composition and the homopolymeric nature of its component strands, poly(dG-dC) differs from DNA and poly[d(A-T)] by its preference for the A-conformation, although variable portions coexist in the B-form (12). Fig. 3 depicts the B-conformation of DNA in aqueous, and the A-conformation in dehydrated, environments.

Stable binding of MG requires double-stranded DNA in the B-conformation. This is proved by observations that ethanol at graded concentrations releases MG from DNA. Effects of ethanol on DNA in solution have been extensively studied*.

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*From 20 vol per cent, ethanol begins to denature DNA as indicated by a sharp drop in viscosity which decreases further with increasing alcohol concentrations (13). Up to 40 vol per cent, ethanol decreases the median denaturation temperature, T_m, of DNA, indicating progressive denaturation; but above 40 vol per cent ethanol, T_m increases again owing to the beginning of the B $\rightarrow$ A transition (14). At 65-78 vol per cent ethanol, DNA exhibits (by circular dichroism) the conformational change from the B- to the A-conformation (15). In 100 per cent ethanol, DNA is completely denatured (16).

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FIG. 3. Schemes of the A and B forms of DNA.

A-form   B-form
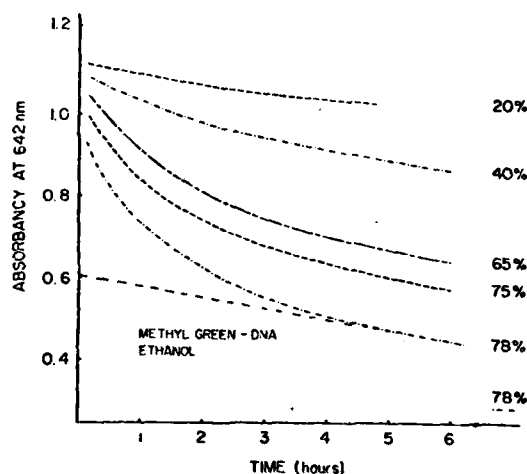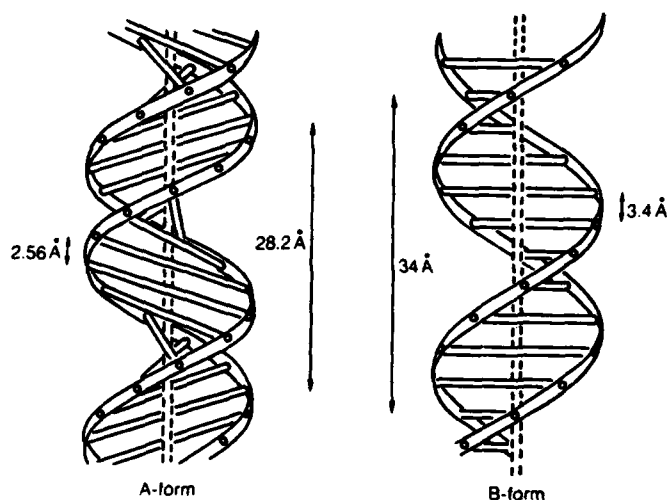
2.56 Å   28.2 Å   34 Å   3.4 Å

FIGURE 4 Effect of ethanol on the Methyl Green-DNA complex. Time courses for the release of Methyl Green from its complex with DNA were measured at different volume percents of ethanol; for 78% alcohol the extrapolation to zero time of the linear portion of the release kinetics is shown. Concentrations: 106 µg/ml of MG-DNA complex.

The effects of ethanol from 20 to 78 vol per cent on the DNA-MG complex are shown in Fig. 4. Increasing ethanol concentrations liberate MG at increasing rates whose initial curvatures show transition to linearity beyond 4.5 hrs (i.e. the time required to decolorize free MG). When the curved portions of the release kinetics were corrected by subtracting the contribution of the linear process, it was discovered that the course of MG liberation was a composite of the linear rate and of an initial burst of release of the dye, followed by a first order decay such as is seen for free MG. Apparently, the slow linear liberation of the dye was the result of progressive denaturation of DNA, while the sudden release of the bulk of MG was caused by the B → A transition of DNA. M G was not extracted from DNA by ethanol: placing a sample of the solid DNA-MG compound into 100 per cent ethanol did not decolorize it upon prolonged exposure.

The forces that bind MG to DNA are predominantly electrostatic: Mg^{2+} at concentrations from 10^{-3} to 5×10^{-2} displaces MG from DNA at rates and to endpoints shown in Fig. 5. In contrast, urea at 6 M (having a pH of 7.2) displaces only 38 per cent of MG from DNA.
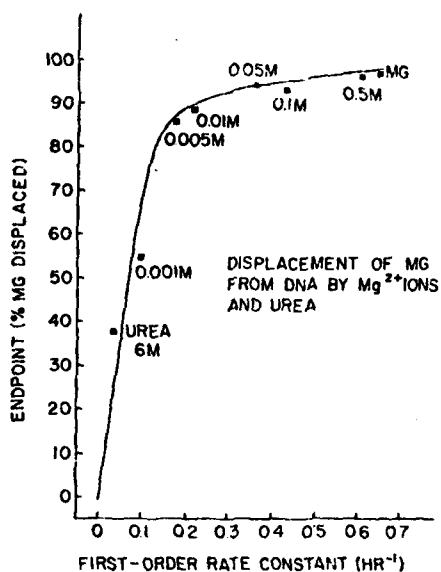
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FIGURE 5: Displacement of MG from DNA by magnesium ions and urea. Mg^{2+} and urea were used at the concentrations indicated in the diagram; 1.88×10^{-5} M MG was considered bound to 2.45×10^{-4} M DNA phosphorus present.

Urea is known to break nonpolar bonds. The bimodality of the curve in Fig. 5 reveals that ~90 per cent of MG are displaced by a process whose endpoints are strongly dependent upon the rate constant of the displacement reaction while at Mg^{2+} concentrations of $>10^{-2}$ M, the displacement of the remaining MG is much less dependent upon the 1st order rate constants. Apparently, the binding of MG to DNA is bimodal, involving perhaps binding to both strands of the double helix across the minor groove as well as binding to individual strands. Binding to single strands is, indeed, indicated by the reactivity of MG with the DNA of ϕX174 (10).

The electrostatic nature of the interaction of MG with DNA is also illustrated by the effect of the dye on the thermal denaturation profile ("melting curve") of DNA, Fig. 6. MG, acting as a counterion to the DNA phosphates, shifts this profile to higher temperatures (ΔT_m = ~12° C for the stoichiometric complex). Crystal violet which differs from MG only be the absence of a quarternary ammonium nitrogen has no significant effect on the thermal denaturation of DNA (Fig. 6). Evidently, the quarternary amino group of MG is prominently involved in the binding of the dye to DNA.

IV. KINETICS OF DISPLACEMENT OF MG BY DRUGS

Before undertaking the survey of the displacement of MG from DNA by drugs, it was necessary to study the kinetics of the displacement reaction. The test compound was the antimalarial quinacrine (Fig. 7). This drug binds to DNA by intercalation (17) with a stoichiometry of one quinacrine molecule-cule per two base pairs (18). Intercalation unwinds the double helix and causes drastic changes in the molecular architecture of the B-conformation of DNA, foremost a doubling of the intervals between the base pairs at the intercalation
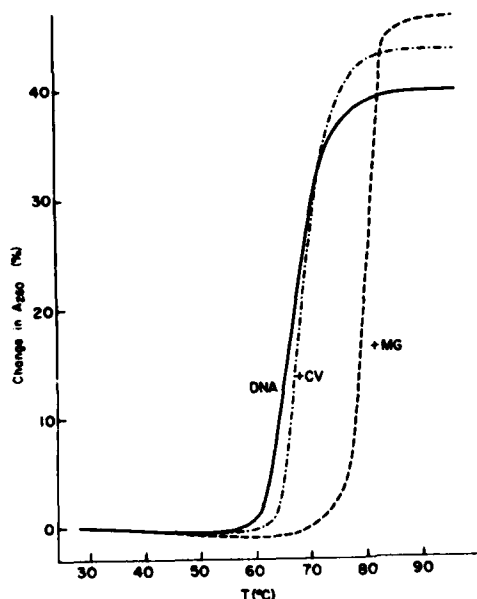
FIGURE 6: Thermal denaturation of 30 μg/ml of calf thymus DNA in the presence of Methyl Green or Crystal Violet and in their absence. Increases in absorbances at 260 nm were calculated with respect to DNAs absorbancy at room temperature. Concentrations of MG and CV, 7.5×10^{-6} M.

Fig. 7. Quinacrine

sites. This may distort the binding sites for MG. Also: the pK_1 (of the tertiary aliphatic amino group, Fig. 7) is ~10 and the pK_2 (of the ring-substituted secondary amino group, Fig. 7) is ~8. Hence, quinacrine exists at physiological pH as a dication and may, like MG^{2+} (Fig. 5), displace MG from DNA by ionic competition. In fact, in addition to intercalation, one quinacrine molecule is bound per 3 nucleotides by electrostatic attraction to the periphery of the double helix (18).

Fig. 8 shows that at a molar ratio of free quinacrine/DNA-bound MG of 5, the displacement reaction is of first order with time, while at a ratio of 1.25 it is of second order. At a "standard" molar ratio of 2.5, the reaction is of first order for the initial 2 hrs of the displacement reaction and then changes to second order kinetics.

These observations can be explained stoichiometrically. Binding of MG to DNA occurs with 0.077 dye molecules per nucleotide, while quinacrine binds to the extent of 0.58 molecules per nucleotide. Since 7.5 more quinacrine binds to DNA than the number of MG molecules which it displaces, it follows that with initially low or critically decreasing concentrations of free quinacrine, these concentrations become one of the rate-limiting factors of the displacement reaction, the other being the concentrations of remaining bound MG. For the screening method reported here, the molar ratio of free drugs to DNA-bound MG was standardized at 2.65 which affords a 3 hr period for determining the first order rate constant of the displacement reaction.
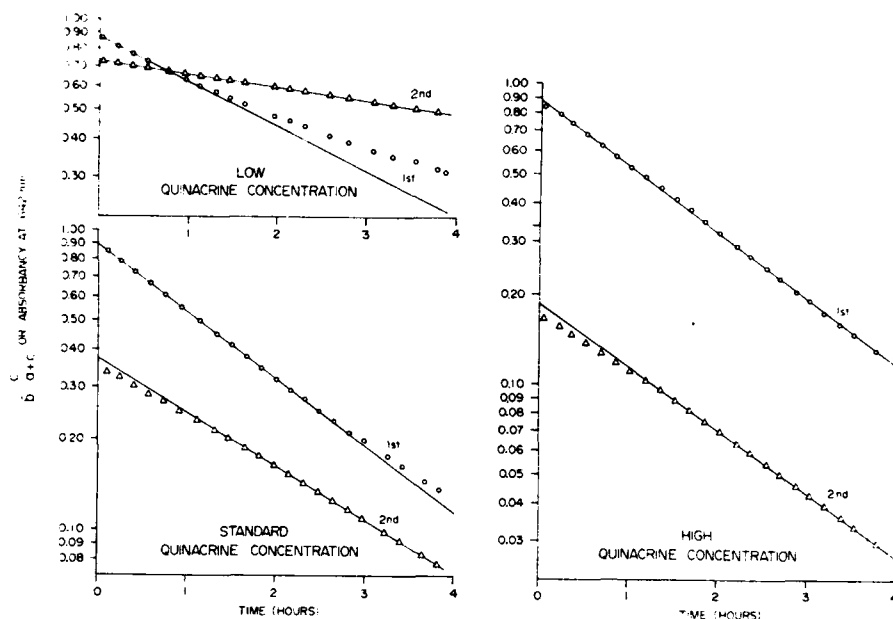
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Fⁱᴳ. 8 •*First-order (1st) and second-order (2nd) kinetics for displacement of methyl green from its complex with DNA by quinacrine*

a = initial concentration of DNA-bound methyl green (18.8 μM; *b* = initial concentrations of quinacrine (50 μM, standard concentration of quinacrine; 100 μM, high; 25 μM, low); *c* = concentration of the amount of methyl green which remained bound to DNA. On the ordinate absorbance is plotted for first-order kinetics, and *c/(b − a + c)*, for second-order kinetics.

The "absolute endpoint" of MG displacement is determined after keeping the reaction mixtures in the dark for one or two days, ascertaining periodically that no more MG is displaced from DNA.

The first order rate constant of the decolorization of free MG is $0.65 \times hr^{-1}$ and has not been attained in any displacement by drugs. Hence, the rearrangement of liberated MG is not a rate-limiting factor in displacement analysis and all measured displacement rates are characteristics of the drugs studied.

V. DISPLACEMENT OF METHYL GREEN FROM DNA BY DRUGS

Results of displacement analyses of MG from DNA by drugs are summarized in Fig. 9. The bimodal correlation between first order reaction constants and absolute endpoints of displacement can be virtually superimposed on the same correlation (Fig. 5), resulting from the displacement of MG by graded concentrations of Mg^{2+}. As in Fig. 5, there is an inflection point at ∿90 per cent displacement. Up to this value, end points are strongly dependent upon the rate constants of the dis-
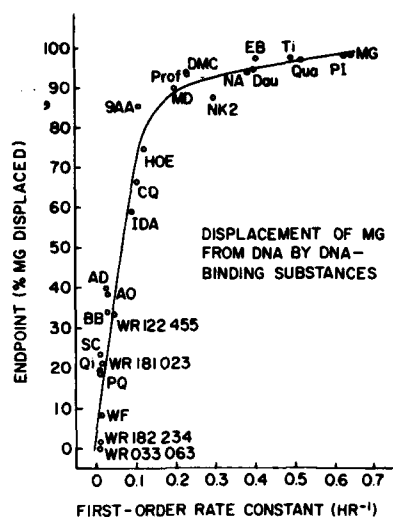
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FIGURE 9. Displacement of MG from DNA by drugs and dyes. Displacing compounds: propidium iodide (PI), quinacrine (Qua), tilerone (Ti), ethidium bromide (EB), daunomycin (Dau), Nitroakridin 3582 (NA), distamycin A (DMC), proflavine (Prof), nitroakridin 2 (NK 2), Hoechst 33258 (HOE), chloroquine (CQ), irehdiamine (IDA), actinomycin D (AD), Acridine Orange (AO), berberine (BB), side chain of quinacrine and chloroquine (SC), quinine (Qi), primaquine (PQ), warfarin (WF), α-(2-piperidyl)-3,6-bis(trifluoromethyl)-9-phenanthrenemethanol hydrochloride (WR 122455), 6-methoxy-8-(4-amino-1-methylbutylamino)lepidine-4HCl·2C$_2$H$_5$OH·H$_2$O (WR 181023), 6-methoxy-8-(4-amino-1-methylbutylamino)quinaldine dihydrochloride (WR 182234), and α-(di-*n*-heptylaminomethyl)-6-bromo-9-phenanthrenemethanol hydrochloride (WR 033063); the displacing compounds were used at a concentration of 5 × 10^{-5} M; 1.88 × 10^{-5} M MG was considered bound to 2.45 × 10^{-4} M DNA phosphorus present; 1-cm light path. Miracil D (MD) and 6-chloro-2-methoxy-9-methylaminoacridine were used at a concentration of 5 × 10^{-6} M; 1.88 × 10^{-6} M MG was considered bound to 2.45 × 10^{-5} M DNA phosphorus present; 10-cm light path.

placement reactions, while for the displacement of the remaining 10 per of MG, the dependence is less marked. The bimodality of displacement again suggests the existence of two discrete binding modes of MG to DNA in addition to differences in the abilities of the tested compounds to interfere with these modes either by ionic competition or by structural distortion of the B-configuration of DNA

The most active displacing drugs in the upper branch of the curve (Fig. 9) are the strong and stoichiometric intercalators, propidium iodide quinacrine, tilorone, ethidium bromide, daunomycin, two nitroacridines, proflavine, and miracil D. The non-intercalating antibiotic, distamycin A, which binds to DNA pseudo-irreversibly (19) is also found in this part of the diagram.

The left branch of the curve lists some non-intercalating substances such as Hoechst 33258, or the aliphatic side chain of quinacrine (and chloroquine) as well as those intercalative agents which bind more weakly to DNA [chloroquine, primaquine (17)] or bind with a lower stoichiometry, owing to steric hindrance (actinomycin D, berberine, quinine, irhediamine).

The phenanthrene derivative WR 122 455 will on structural grounds, be able to intercalate into DNA, but it lacks a ring nitrogen or a substituted amino group for electrostatic attraction to DNA phosphates; the 2-piperidyl substituent is weakly basic (pK 2.8) and is not coplanar with the phenanthrene ring. Intercalation, therefore,

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will be relatively weak, owing to lack of electrostatic anchoring and possible steric hindrance. This should be compared to the strong binding of propidium iodide and ethidium bromide which are phenanthridines. Deletion of the two amino groups from ethidium is known to decrease the DNA binding constant by factors of 10 to 20.

WR 181 023 is a structural analog of primaquine. Fig. 9 shows that the MG displacement parameters are similar to those of primaquine. Both compounds have a low pK_2 of the 8-amino group and, hence, will exist at physiological pH as monoprotonated cations which anchor poorly to DNA. The pK_2 of primaquine is 3.1.

The nature of the low endpoints of MG displacements (<10 per cent) by warfarin and by the experimental substances WR 182 234 and WR 033 063 is not well understood. Warfarin has failed all other tests for interaction with DNA. For practical purposes, endpoints of <10 per cent MG displacement and rates close to 0 should be regarded as non-significant.

VI. CORRELATION OF MG DISPLACEMENT DATA WITH BIOLOGICAL ACTIVITIES

A study of the ability of DNA intercalators to eliminate drug-resistance genes from the R-plasmid R1 (in Salmonella typhimurium) has yielded activities against the kanamycin resistance gene (3) that are directly proportional to the MG displacements of the active drugs, screened by the method reported here. This is documented in Fig. 10 (10).
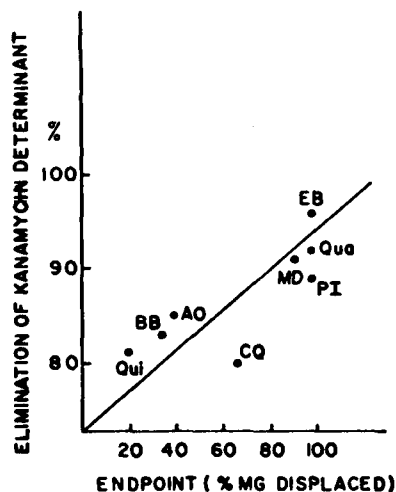

Fig. 10

The results of Fig. 10 are explained by the fact that intercalation binding into plasmid DNA blocks selectively the replication of this DNA so that in growing bacterial cultures plasmid$^+$ cells are continuously being diluted by the plasmid$^-$ progeny (4).

The ability of selected intercalants to facilitate the disassembly of ribosomes in vitro has been measured in terms of the relative rates of this process (20). The diagram, Fig. 11, shows these relative rates as a function of the absolute endpoints of MG displacement (20). The curve exhibits the same bimodality as that shown in Figs. 5 and 9, suggesting that the
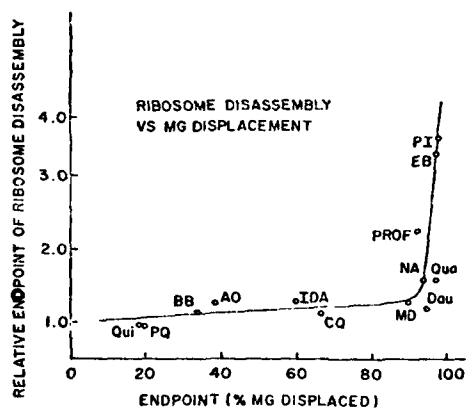
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FIGURE 11 Effect of test compounds on disassembly of ribosomes (Wolfe et al., 1972) as a function of Methyl Green displacing end points.

effects on ribosomes are caused by drug interactions with double-stranded runs of ribosomal RNA (20).

By a differently designed method it was found for a series of planar phthalanilides that concentrations that displaced 25 per cent of DNA-bound MG were directly proportional to antibacterial concentrations of the same compounds (21). This has been the first contribution of a form of MG displacement analysis to a program in experimental chemotherapy.

A comparison of arbitrary endpoints (at 18 hrs) of MG displacements by graded concentrations of proflavine, actinomycin D, and anthramycin has shown anthramycin to be the least active compound, although it was an effective inhibitor of DNA and RNA (but not protein) biosyntheses (22). Anthramycin binds to DNA slowly without intercalation (23).

Semi-quantitative estimation of MG displacement has been used to select 19 substituted N-heterocyclic compounds as potential immunosuppressive agents in the graft vs. host reaction that results from the development of cellular immunity (24). Seven of these preselected compounds, i.e. 37 per cent, showed significant immunosuppressive activity (24). Since they were tested at different concentrations, the numerical results do not lend themselves to regression analysis. It is noteworthy, however, that a MG displacement prescreen predicted correctly pharmacological activity of 37 per cent of a screened population of compounds.

After the present screening method had been applied to the study of the binding of the antibiotic, distamycin A, to DNA (25), the method was used to pilot the develoment of distamycin congeners with graded numbers of N-methylpyrrole rings in the molecule (26). Increasing numbers of N-methylpyrrole constituents correlated with increasing displacement of MG (26) and with increasing inhibitions of the DNA polymerase reactions of Escherichia coli and of Rous sarcoma virus (rev. in 27).

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Finally, MG displacement has been used to discover the presence of DNA-complexing compounds in crude fermentation mixtures of an antibiotics search program. Aliquots of such unresolved mixtures were added directly to solutions of the MG–DNA reagent, and several DNA-binding antibiotics were isolated from those mixtures which had displaced MG from DNA (28).

VII. DISCUSSION AND SUMMARY

The principal result, reported in this article, is that spectrophotometric analysis of the displacement of MG from its complex with double-stranded DNA by DNA-binding clinical and experimental drugs yields reaction rates and absolute endpoints which are correlated to biochemical and chemotherapeutic activities of the tested compounds, when these activities are caused by DNA template toxicity. Tables 1 and 3 document that this mechanism of action is prevalent among antiparasitic and anticancer drugs.

Structural rules for the design or the recognition of intercalating DNA complexers are well developed (5) but MG displacement analysis yields numerical indications of relative affinities and/or stoichiometries of DNA binding, as discussed in Section V.

Structural rules for peripheral (i.e. non-intercalative) binding to DNA are not so well developed, and MG displacement can serve as a facile indication that such binding occurs (25).

Finally, the ability of fermentation beers from antibiotics search programs to displace MG will signal the presence of DNA-binding compounds of completely unknown structures and facilitate their isolation (28).

Structural variation of DNA binding drugs can be piloted by MG displacement analysis (21, 26) in the expectation that enhanced MG displacement will signal enhanced chemotherapeutic activity.

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